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13. ABSTRACT (Maximum 200) Basic fibroblast growth factor (bFGF, FGF-2) is a classical mitogen in endothelial cells and an important angiogenesis factor in breast cancer. Although it acts as a transforming factor in fibroblasts, its expression in breast cancer is associated with differentiation and a better prognosis. We introduced bFGF into two breast cancer cell lines at different ends of the dedifferentiation spectrum. Relatively differentiated malignant MCF-7 cells were growth inhibited by bFGF expression and were able to export all isoforms of transfected bFGF but highly dedifferentiated MDA-MB-231 cells were not inhibited and did not export bFGF. MCF-7 cells expressing and exporting nuclear localizing bFGF isoforms had decreased FGF receptor 4, decreased binding by exogenous bFGF and were unable to phosphorylate FGFR1 and MAP kinase. While levels of p27 <sup>KIP1</sup> were elevated, G <sub>1</sub> cell cycle inhibition did not correlate with inactivation of cdk2 or dephosphorylation of RB, suggesting an alternate, possibly direct nuclear mechanism. The expression of bFGF inhibited anchorage independent growth in soft agar in both cell lines, with a much greater efficiency in MCF-7 cells. Expression of the 18 kD bFGF isoform also inhibited migration and invasion in Matrigel by MDA-MB-231 cells. These data demonstrate for the first time that expression of bFGF induces a more differentiated phenotype in breast cancer cells.					
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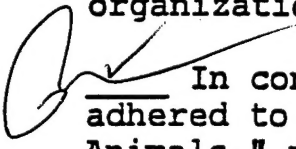
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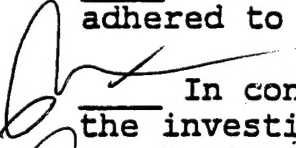
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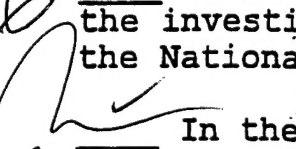
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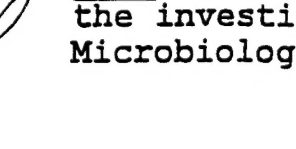
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**THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN HUMAN BREAST CANCER**Annual report, September 30, 1998**INTRODUCTION**

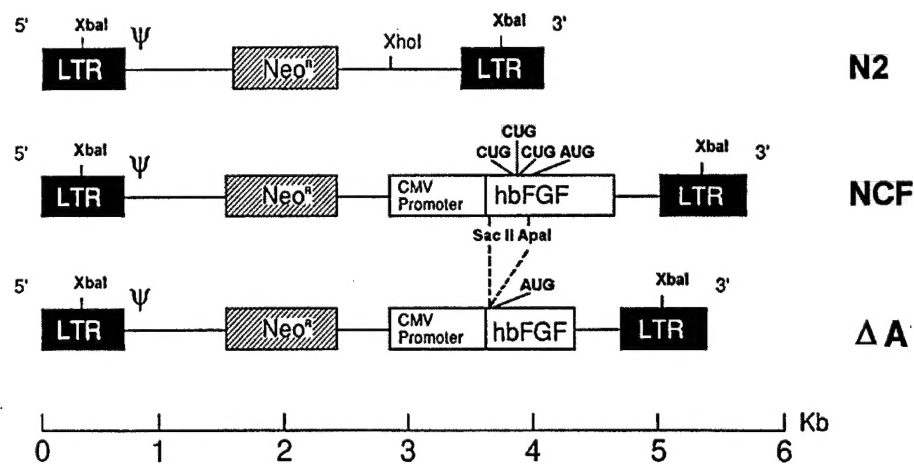
Basic fibroblast growth factor (bFGF, FGF-2) is a heparin binding polypeptide growth factor that exhibits diverse functions in different cell types (1). In the development of breast cancer, the intracellular bFGF content of mammary epithelial cells decreases to undetectable levels as these cells become malignant. This decline is due to extracellular secretion (2) and subsequent cessation of synthesis (3, 4). The secreted bFGF acts as a key angiogenesis factor in breast cancer (2), an effect due to its mitogenic effects on endothelial cells (5). In contrast to its effects on endothelial cells, however, the effects of bFGF on breast cancer cells can be paradoxical (6, 7). We have demonstrated that in MCF-7 human breast cancer cells, bFGF binds to high affinity receptors, activates MAP kinase (7) and induces the mitogenic  $G_1$  events. However, it also induces higher levels of  $p21^{WAF1/CIP1}$ , inactivates cdk2 and dephosphorylates Rb, effects that result in a net inhibition of proliferation and segregation of the cells in the  $G_0/G_1$  phase of the cell cycle (8).

Data from a number of studies have demonstrated that the presence of bFGF is associated with a less malignant phenotype in breast cancer. Fluids aspirated from "apocrine" cysts lined with premalignant metaplastic epithelial cells have lower FGF content than cysts lined with flattened epithelial cells of low malignant potential (9). In infiltrating ductal carcinomas, bFGF was observed only in benign myoepithelial remnants and basement membranes surrounding the tumors, but not in the malignant cells (10). Significantly less bFGF mRNA was present in breast cancer biopsies than in non-malignant breast biopsies (3, 4). Similarly, bFGF was expressed in normal mammary epithelium-derived cell lines, while it was either absent or detectable at low levels in malignant cell lines ZR-75-1, MCF-7, T-47D, and MDA-MB-231 (3). We noted that nontransformed immortalized mammary epithelial cell lines that contained measurable bFGF levels had slower rates of proliferation than breast cancer cells that did not have measurable bFGF levels (Wieder, R., unpublished observations). However, a causative role for intracellular bFGF in the diminished proliferative rates has not been established. We undertook this study to determine if expression of bFGF in MCF-7 cells can inhibit their proliferation rates and whether the mechanism of growth inhibition induced by intracellular bFGF is the same as that responsible for growth inhibition by exogenous bFGF (8). Our data demonstrate that MCF-7 cells permanently transduced with a variety of bFGF gene constructs overexpress bFGF and exhibit decreased proliferative activity. However these effects do not correlate with induction of MAP kinase phosphorylation or upregulation of  $p21^{WAF1/CIP1}$ , events that vary with the isoforms of bFGF overexpressed and the activity of FGF receptors.

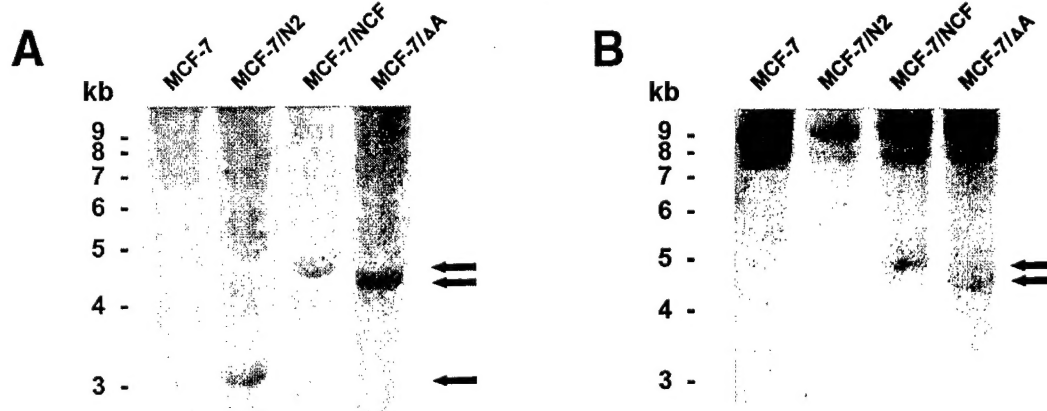
We also constructed bFGF-expressing MDA-MB-231 cells, a poorly differentiated, estrogen independent, highly invasive breast cancer cell line homozygous for mutant p53, to determine if expression of bFGF can reverse malignant features of breast cancer cells. We demonstrate, for the first time that, consistent with the clinical observations of better prognostic outcomes of patients with breast tumors that contain bFGF, breast cancer cells overexpressing bFGF have a less malignant phenotype in in vitro transformation assays.

**BODY****MCF-7 breast cancer cells engineered to express bFGF secrete all isoforms**

We constructed MCF-7-derived cells expressing human bFGF using retroviral gene transfer with N2-based vectors coding for the 18 kD cytoplasm localizing ( $\Delta A$ ) or the 18, 22 and 24 kD species (NCF) containing both cytoplasm and nucleus localizing bFGF species (figure 1), and selected the cells in G418 (11). Southern blot analysis of restriction endonuclease XbaI-digested genomic DNA probed with a bFGF cDNA fragment showed that intact vector DNA of the expected sizes of 3257, 4955 and 4648 bp were present in MCF-7/N2, MCF-7/NCF<sub>FGF(18,22,24)</sub> and MCF-7/ $\Delta A$ <sub>FGF(18)</sub> cells, respectively (figure 2).

**Figure 1**

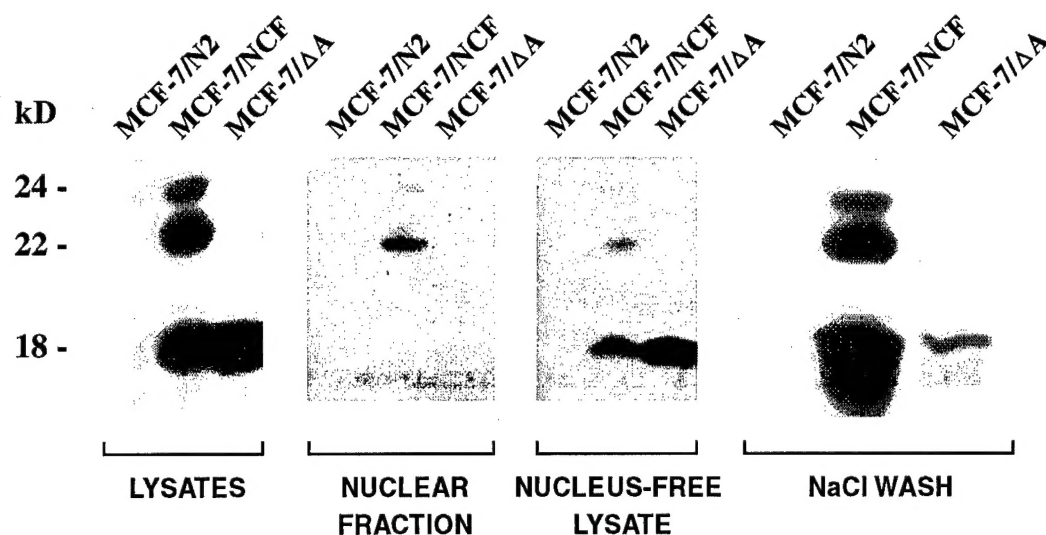
**Figure 1.** Retroviral vectors used to transduce MCF-7 cells. NCF contains an immediate-early CMV promoted human *bFGF* cDNA cloned into the Xho I site of N2. A Sac II/Apa I fragment was deleted from NCF to construct  $\Delta A$ , as described (11).

**Figure 2**

**Figure 2.** Southern blot analysis of restriction endonuclease Xba I-cleaved genomic DNA from MCF-7 cells transduced with N2, NCF and  $\Delta A$ . Ten  $\mu$ g restriction endonuclease-digested DNA samples were electrophoresed in 1% agarose, transferred to nylon membranes and probed with A. a  $^{32}$ P-labeled 526 bp Xma III-Nco I *Neo* fragment of N2, denatured and B. rehybridized with a  $^{32}$ P-labeled 432 bp endonuclease Bam H1 fragment of *bFGF* cDNA spanning positions -27 to +406 of the cDNA coding sequence. Arrows identify the 3257, 4955 and 4648 bp Xba I fragments in MCF-7/N2, MCF-7/NCF<sub>FGF(18,22,24)</sub> and MCF-7/ $\Delta A$ <sub>FGF(18)</sub> cells, respectively.

Western blots demonstrated that these cells expressed the expected species (figure 3). Subcellular fractionation studies, carried out as described by Renko, et al. (12) demonstrated that, as expected, MCF-7/NCF<sub>FGF(18,22,24)</sub> cells had DNA localize both in the cytoplasm and the nucleus while MCF-7/ $\Delta$ A<sub>FGF(18)</sub> cells had bFGF that only localized in the cytoplasm. 2 M NaCl washes of the cell monolayers demonstrated that all isoforms of bFGF are secreted by MCF-7 cells, in contrast to NIH 3T3 cells that are only capable of exporting the 18 kD species (13) (figure 3). Subcellular localization was confirmed using immunofluorescence photography with FITC labeled secondary antibodies (figure 4).

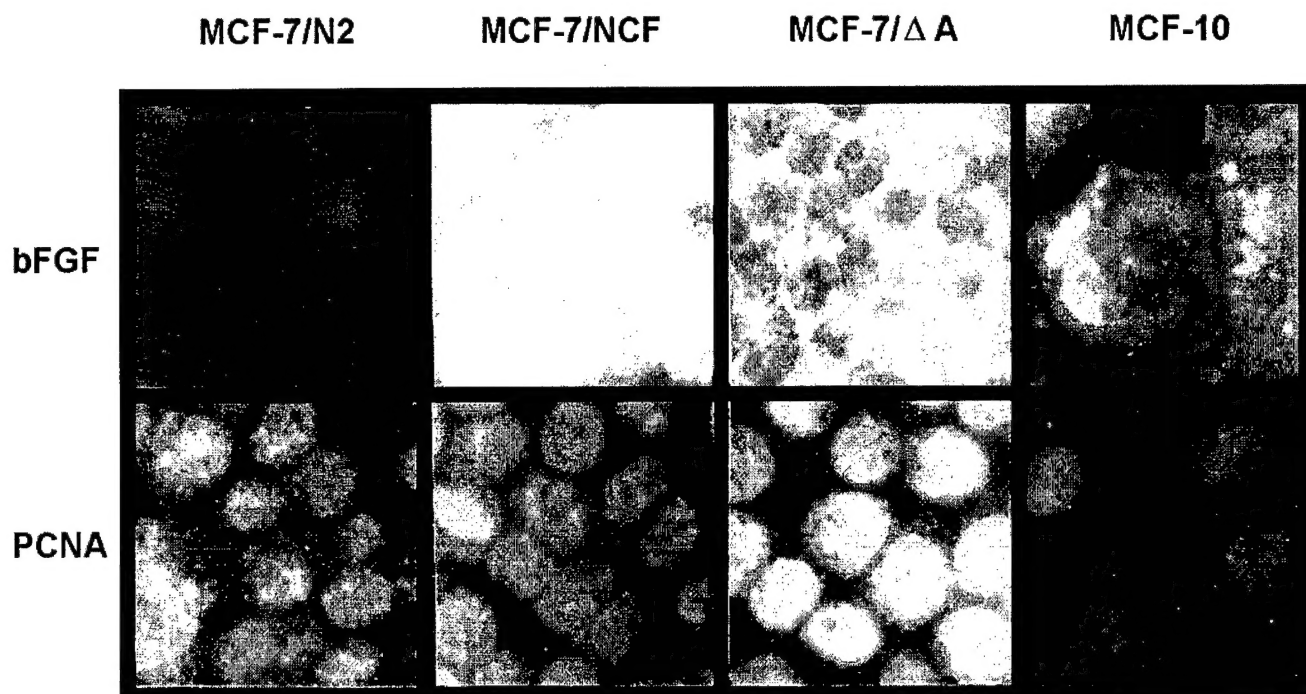
Figure 3



**Figure 3.** Western blots demonstrating expression, nuclear and cytoplasmic distribution and secretion of bFGF species expressed in the transduced cells. MCF-7/N2 cells contain the parental vector, MCF-7/NCF<sub>FGF(18,22,24)</sub> express the 18 kD, the 22 kD doublet and the 24 kD bFGF moieties and MCF-7/ $\Delta$ A<sub>FGF(18)</sub> cells express only 18 kD bFGF. Cell lysates were prepared, and 100  $\mu$ g protein were electrophoresed per lane. Subcellular fractionations were carried out as described (12). Nuclear preparations from three confluent tissue culture plates were washed twice with buffer containing 1% NP40 and electrophoresed in a 12.5% SDS PAGE gel system along with cellular lysates from which nuclei were removed. Export was determined by 2 M NaCl washes of confluent MCF-7-derived cells rendered quiescent by a 48 hour incubation in serum-free medium, precipitated with 10% TCA and electrophoresis in a 12.5% SDS PAGE gel.



Figure 4



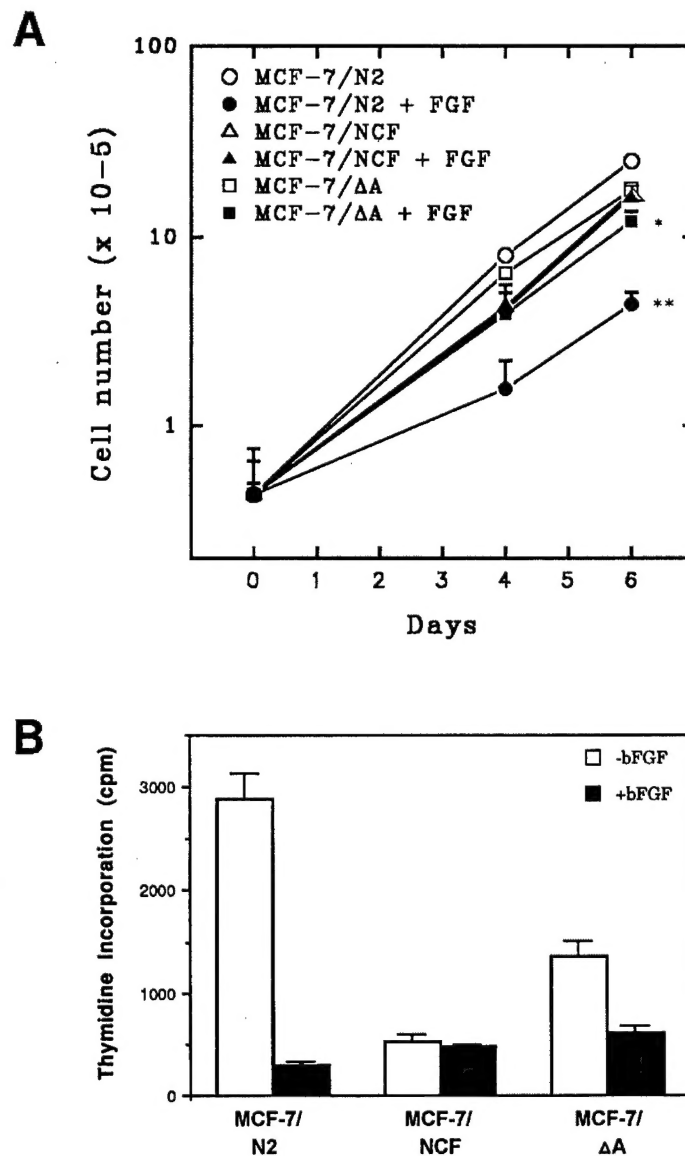
**Figure 4.** Immunofluorescence of MCF-7/N2, MCF-7/NCF<sub>FGF(18,22,24)</sub>, MCF-7/ΔA<sub>FGF(18)</sub> and MCF-10 cells stained with monoclonal antibody to bFGF or antisera to PCNA, used as a localization control. Photographs were taken at 400 X magnification and demonstrated the presence of both nuclear and cytoplasmic immunoreactive bFGF in MCF-7/NCF and MCF-10 cells and exclusively cytoplasmic immunoreactive bFGF in MCF-7/ΔA cells. Anti-PCNA antibody was used as a nuclear localization control.

#### Expression of bFGF inhibits proliferation of MCF-7 cells

Cells expressing the various isoforms of bFGF had significantly reduced proliferation rates (figure 5A) and decreased thymidine uptake (figure 5B) than controls. Addition of 0.5 ng/ml recombinant bFGF to the medium resulted in significant inhibition of growth in MCF-7/N2 cells ( $p < 0.0005$ ) and MCF-7/ΔA<sub>FGF(18)</sub> cells ( $p < 0.005$ ) but did not have an effect on MCF-7/NCF<sub>FGF(18,22,24)</sub> cells (figure 5A). Addition of a neutralizing anti-bFGF antibody, previously shown to restore the growth of MCF-7 cells incubated with 0.5 ng bFGF/ml (Fenig, et al., 1997), had no effect on restoring the growth rate of MCF-7/NCF<sub>FGF(18,22,24)</sub> cells (data not shown). Similar patterns of response were observed in each cell line when <sup>3</sup>H-thymidine incorporation was evaluated (figure 5B). Overexpression of bFGF was also associated with an increase in the fraction of cells in the G<sub>1</sub> phase of the cell cycle. Cell cycle distribution studies showed that 31.7% of MCF-7/N2 cells obtained from subconfluent cultures were in G<sub>0</sub>/G<sub>1</sub> and 49.7% were in S phase. MCF-7/NCF<sub>FGF(18,22,24)</sub> and MCF-7/ΔA<sub>FGF(18)</sub> cells had 55.1% and 60.3% of cells in G<sub>1</sub>, and 33.4% and 27.1% in the S phase, respectively. Addition of exogenous recombinant bFGF (1 ng/ml) increased the percentage of cells in G<sub>1</sub> in control MCF-7/N2 cells from 31.7% to 69.9%, but only minimally increased the fraction of G<sub>1</sub> MCF-7/ΔA<sub>FGF(18)</sub> cells from 60.3 to 65.0%. Similar treatment of MCF-7/NCF<sub>FGF(18,22,24)</sub> cells did not increase the fraction of G<sub>1</sub> cells.



Figure 5



**Figure 5.** Effect of the overexpression of bFGF on the growth and response to exogenous bFGF in MCF-7 cells. A. Proliferation kinetics.  $4.3 \times 10^4$  MCF-7 cells transduced with NCF, ΔA or with N2 as a control were incubated in DME and 5% FCS  $\pm$  500 pg/ml bFGF in 100 mm tissue culture plates. The medium with fresh bFGF (in appropriate plates) was changed every 48 hours. Viable cells were manually counted in a hemocytometer in 0.2% trypan blue. Cells delineated MCF-7/NCF and MCF-7/ΔA express bFGF moieties described in figure 2 and MCF-7/N2 cells contained the parental vector. \* significant difference to  $p < 0.005$  compared with MCF-7/ΔA; significant difference to \*\*  $p < 0.0005$  compared with MCF-7/N2 (Student's T test). B. Thymidine incorporation.  $1.6 \times 10^3$  MCF-7-derived cells were incubated in 24 well plates, one microcurie ( $\mu$ Ci) of [methyl- $^3$ H] thymidine was added to each well on day 4 for 2 hours and tritium incorporation was measured, as described.

### Expression of bFGF in MCF-7 cells inhibits bFGF binding and downregulates FGFR4

To evaluate whether the growth inhibition of cells overexpressing bFGF and their lack of response to exogenously added bFGF are associated with alterations in FGF receptor levels,  $^{125}\text{I}$ -bFGF binding studies and crosslinking experiments with high-affinity membrane receptors were performed. The binding studies showed that MCF-7/N2 cells had a bFGF affinity constant ( $K_d$ ) of  $135.7 \pm 112.1$  pM and an estimated number of  $2606 \pm 363$  high affinity binding sites per cell. Both MCF-7/ $\Delta A_{\text{FGF}(18)}$  and MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells exhibited diminished bFGF binding at low concentrations of bFGF, such that a  $K_d$  could not be calculated. At a saturating bFGF concentration of 0.5 ng/ml, binding to MCF-7/ $\Delta A_{\text{FGF}(18)}$  and MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells was diminished to 68% and 42% of that observed in MCF-7/N2 cells, respectively.

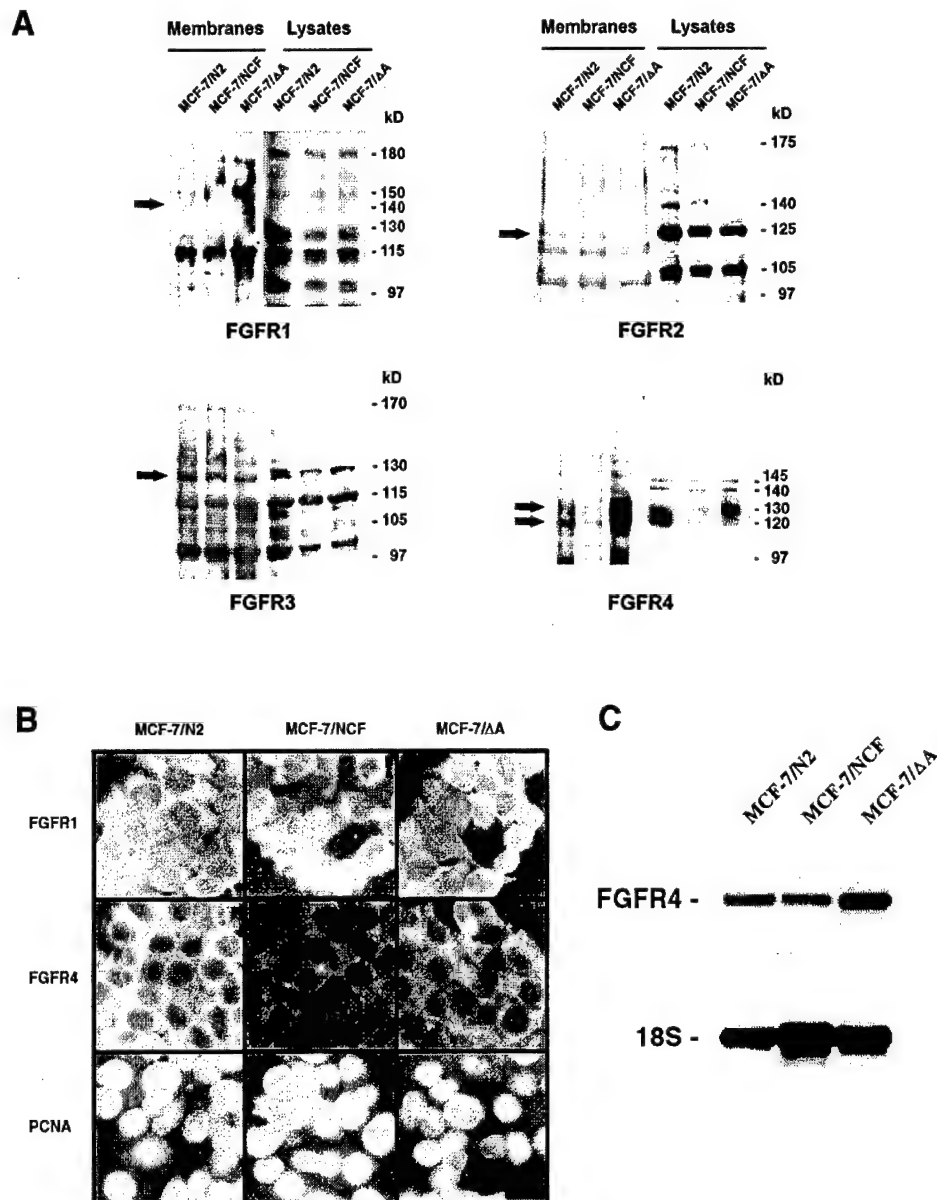
Crosslinking of  $^{125}\text{I}$ -bFGF to high-affinity bFGF receptors was carried out with cells grown to confluence.  $^{125}\text{I}$ -bFGF was crosslinked using DSS, and lysates were subjected to polyacrylamide gel electrophoresis and autoradiography. A 160 kD  $M_r$  complex was found to contain the  $^{125}\text{I}$ -bFGF in MCF-7/N2 cells. Incubation with 100-fold excess of unlabeled bFGF abolished the radioactive labeling of the complex (data not shown). This 160 kD crosslinked complex is interpreted as a receptor mass of 142 kD crosslinked to the 18 kD bFGF molecule. No evidence of crosslinking of  $^{125}\text{I}$ -bFGF was found in MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells.

While demonstrating decreased binding of exogenously added bFGF to transduced cells, these studies did not distinguish between the possibility that the FGF receptors were occupied due to autocrine binding of bFGF secreted from transduced cells versus alterations in the abundance of the high affinity FGF receptors due to a greater rate of internalization and degradation, altered gene expression, structure or function.

To determine if expression of bFGF modulated the cellular content of FGF receptors that could lead to altered binding by exogenous bFGF, Western immunoblots of cellular lysates were carried out with antibodies to the four FGF receptors in the transduced cell lines. No differences were observed among the three cell types in the total cellular content of FGF receptors 1-3, but the level of FGFR4 was diminished in MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells (figure 6A). Multiple bands, corresponding to various glycosylated forms of the receptors, were evident (14). To determine if the decreased binding by 18 kD recombinant bFGF was due to decreased membrane-association of any of the four FGF receptors in the transduced cells and if the decreased levels of total cellular FGFR4 corresponded to decreased membrane associated FGFR4, subcellular fractionation experiments were carried out (12). The membrane fraction was electrophoresed and immunoblotted with antibodies to each of the four FGF receptors. Figure 6A demonstrates that FGFR4 was diminished in the membrane fraction of MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells when compared to MCF-7/N2 and MCF-7/ $\Delta A_{\text{FGF}(18)}$  cells, consistent with the previously demonstrated decrease in total cellular FGFR4 content in these cells. No differences in the content of any of the other three FGF receptors were found in the membrane fractions of any of the three cell types.

To confirm the decrease in total and membrane-associated FGFR4 in MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells, the cells were stained with immunofluorescent antibodies. Figure 6B demonstrates equivalent staining with antibody to FGFR1 in all three cell types, but markedly reduced staining with antibody to FGFR4 in MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells when compared with either MCF-7/N2 or MCF-7/ $\Delta A_{\text{FGF}(18)}$  cells. Immunostaining with anti-PCNA antibody as a control demonstrated equivalent nuclear staining among the three cell types. To determine if the decrease in FGFR4 was due to a decrease in mRNA levels as a result of overexpression of nucleus-localizing species or whether the effect was seen only at the level of protein, Northern blot analysis was carried out on MCF-7/N2, MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  and MCF-7/ $\Delta A_{\text{FGF}(18)}$  total cellular RNA. Figure 6C demonstrates that the FGFR4 mRNA levels are only slightly decreased in MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells. This suggests that the downregulation of total and membrane-bound FGFR4 in cells expressing all three moieties of bFGF is primarily at the level of receptor protein but that synthesis of FGFR4 may also be partially affected.

Figure 6

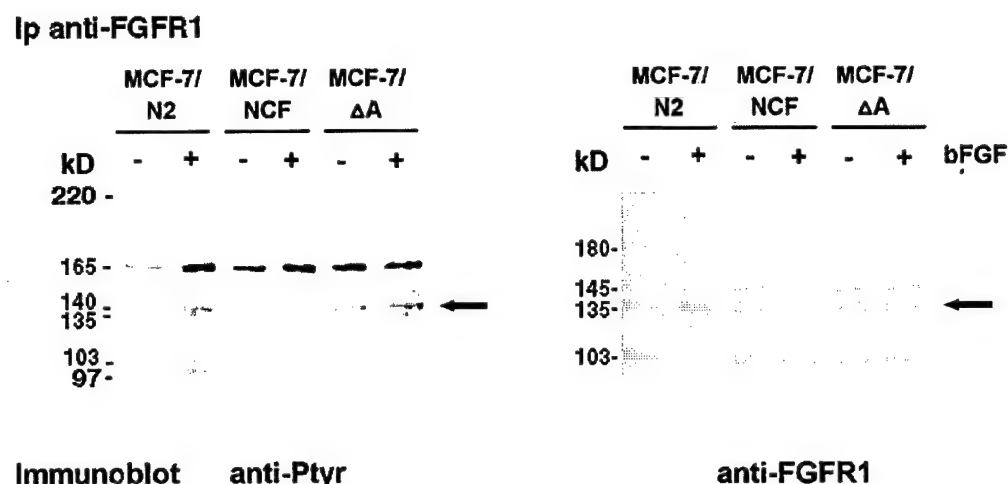


**Figure 6.** A. Western blots of membrane fractions and of total cellular lysates of MCF-7/N2, MCF-7/NCF and MCF-7/ΔA cells stained with antibodies to FGF receptors 1-4. Arrows indicate the functional FGFR species identified in the literature and by immunoprecipitations and Western blots. B. Immunofluorescence of MCF-7/N2, MCF-7/NCF and MCF-7/ΔA cells stained with monoclonal antibody to FGFR1 or antisera to FGFR4 or PCNA, used as a control. Photographs were taken at 400 X magnification, as described, and demonstrated decreased immunoreactive FGFR4 in MCF-7/NCF cells. Non-immune goat IgG was used as a negative control (not shown) C. Northern blots of poly A mRNA with an FGFR4 cDNA fragment probe demonstrating the expression of FGFR4 in all three cellular constructs. Ribosomal 18S RNA probed with an Eco RI cDNA fragment was used as a loading control. The data are from one of three representative experiments.

### Expression of bFGF species differentially affects FGFR and MAP kinase phosphorylation

FGF binding to receptors causes homo- and heterodimerization among the four FGF receptors, resulting in transphosphorylation and initiation of receptor-specific signal transduction pathways (15). Experiments were carried out to determine the phosphorylation of FGFR1 in the various MCF-7 cell constructs. Immunostaining the anti-FGFR1 immunoprecipitates with antibody to phosphotyrosine demonstrated a constitutive phosphorylation of the 140 kD band of FGFR1 in MCF-7/ $\Delta A_{\text{FGF}(18)}$  cells (figure 7). While there was a heavily phosphorylated 165 kD band in all of the cells, the 140 kD FGFR species was the one identified to bind exogenous bFGF in the crosslinking experiments. Exogenous bFGF caused phosphorylation of this band in MCF-7/N2 cells and slightly increased its phosphorylation in MCF-7/ $\Delta A_{\text{FGF}(18)}$  cells. However, there was no phosphorylation of this band in MCF-7/ $\text{NCF}_{\text{FGF}(18,22,24)}$  cells, either constitutively or upon addition of exogenous bFGF. Immunostaining of the membranes with the same antibody to FGFR1 used in the immunoprecipitations demonstrated an equal presence of the FGFR1 in all lanes (figure 7). We investigated one downstream effect of receptor tyrosine activation in these cells, that of MAP kinase phosphorylation.

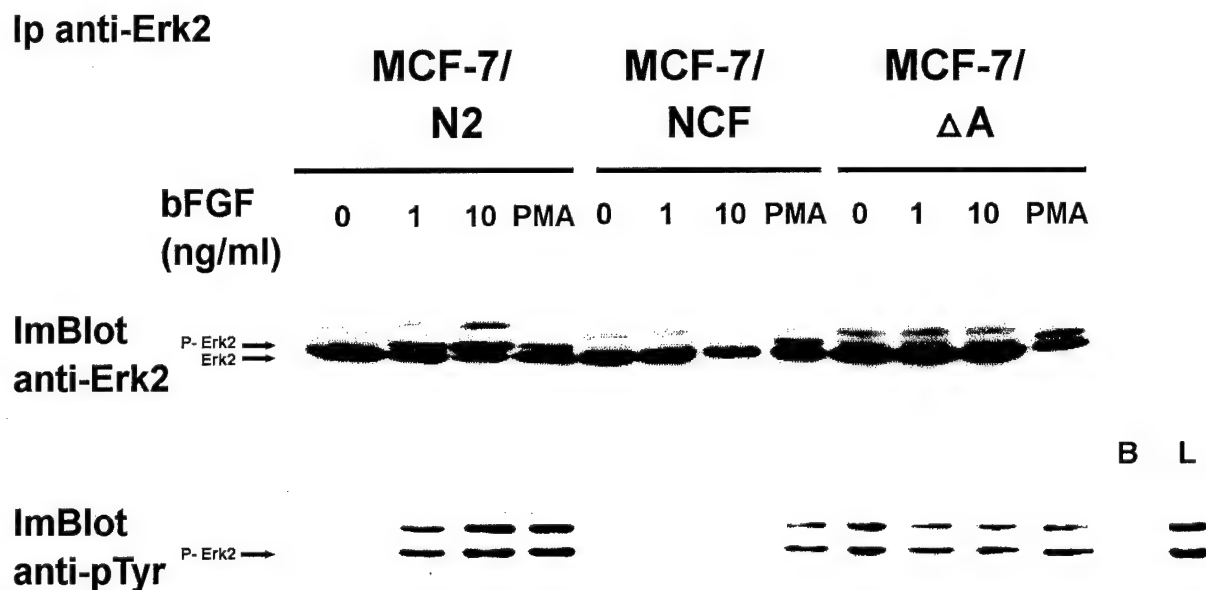
Figure 7



**Figure 7.** Western immunoblot of immunoprecipitates obtained with antibody to FGFR1. The immunoblot membranes were stained with an anti-phosphotyrosine antibody and with the same antibody to FGFR1 used in the immunoprecipitations. The data demonstrate bFGF isotype specific phosphorylation of the 140 kD FGFR1 isoform that bound to  $^{125}\text{I}$ -bFGF (arrow) and an equal presence of the FGFR1 in all lanes.

To determine MAP kinase activation, ERK2 was immunoprecipitated from cell lysates before and after a 15 minute incubation with bFGF using an anti-ERK2 monoclonal antibody and Western blots of the immunoprecipitated proteins were immunostained with monoclonal antibodies to either ERK2 or phosphotyrosine. Figure 8 demonstrates that a 15 minute incubation with bFGF or PMA induced the phosphorylation of ERK2 in MCF-7/N2 cells. ERK2 was constitutively phosphorylated in MCF-7/ $\Delta A_{FGF(18)}$  cells at baseline and was not additionally phosphorylated by bFGF or PMA stimulation. ERK2 was not constitutively phosphorylated in MCF-7/NCF<sub>FGF(18,22,24)</sub> cells, nor was it phosphorylated in response to incubation of the cells with exogenous bFGF. However, stimulation of these cells with 100  $\mu$ M PMA (figure 8) or bovine pancreatic insulin (1  $\mu$ g/ml for 15 minutes; data not shown) resulted in ERK2 phosphorylation, indicating that they were capable of activating MAP kinase but did not generate this response upon bFGF stimulation. While the anti-ERK2 monoclonal antibody also precipitated ERK1, the crossreactivity was not consistent and thus ERK1 activation can not be assessed from this data. The pattern of activation of ERK2 by bFGF correlated with that observed for the tyrosine phosphorylation of FGFR1 and supported our observations that different bFGF species induced different signalling through FGF receptors in MCF-7 cells. These data also demonstrated that the growth inhibitory effects of nucleus-localizing bFGF species overexpressed in MCF-7 cells were not correlated with phosphorylation of ERK1.

Figure 8



**Figure 8.** Western immunoblot of ERK2. Extracts were immunoprecipitated with an anti-ERK2 monoclonal antibody from MCF-7/N2, MCF-7/NCF and MCF-7/ $\Delta A$  cells treated with 0, 1.0 and 10. ng/ml bFGF or 100  $\mu$ M PMA for 15 minutes and were immunostained with anti-ERK2 antibody or with anti-phosphotyrosine antibody. The two blots represent two separate experiments. Arrows identify the unphosphorylated and phosphorylated isoforms of ERK2. Abbreviations B and L denote a control precipitation using only beads or total cellular lysate from bFGF treated cells, respectively.

Cell cycle inhibition by overexpression of bFGF in MCF-7 cells is not mediated through the G<sub>1</sub> cyclins and associated kinase inhibitors

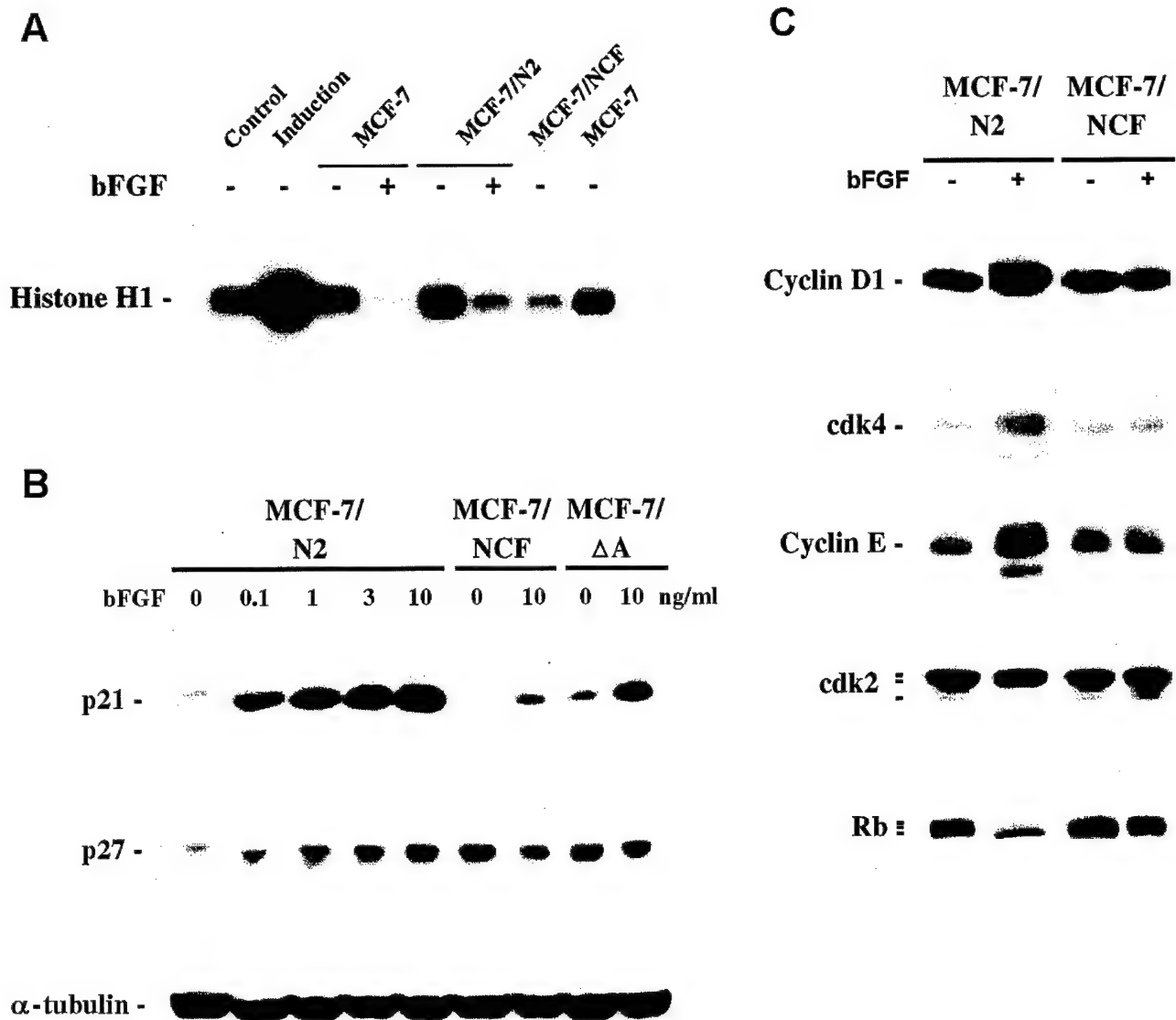
To determine if the inhibitory effects of bFGF overexpressed in MCF-7//NCF<sub>FGF(18,22,24)</sub> cells were mediated through the function of G<sub>1</sub> cyclins, we assayed these cells for the induction of a transferrable inhibitor of cyclin dependent kinases (Figure 9A). The induction of inhibitory activity by bFGF was determined using histone H1 kinase assays with reconstituted cyclin/cdk complexes, as described previously (8). The background kinase activity of unreconstituted mink lung epithelial cell Mv1Lu lysates (control lane) markedly increased upon reconstitution of the cyclin/cdk complexes with exogenous cyclin E (induction lane). Transfer of cellular lysates of MCF-7 cells or MCF-7/N2 cells determined the baseline kinase activity of reconstituted complexes mixed with lysates of cells acting as controls (MCF-7 - bFGF and MCF-7/N2 - bFGF). Lysates from MCF-7 and MCF-7/N2 cells treated with bFGF 10 ng/ml for 24 hours inhibited the kinase activity of cyclin E/cdk complexes (MCF-7 + bFGF and MCF-7/N2 + bFGF) or of cyclin A/cdk complexes (not shown). Lysates from MCF-7/NCF<sub>FGF(18,22,24)</sub> cells transferred to reconstituted complexes also induced a decrease in the histone kinase activity of reconstituted cyclin E/cdk2 complexes, suggesting that a transferrable heat-stable inhibitor was present in the lysates obtained from MCF-7/NCF<sub>FGF(18,22,24)</sub> cells. We proceeded to assay the effect of bFGF expression on p21<sup>WAF1/CIP1</sup>, a heat stable cyclin dependent kinase inhibitor induced by recombinant bFGF in MCF-7 cells (8).

Figure 9B demonstrates that MCF-7/ $\Delta$ A<sub>FGF(18)</sub> and MCF-7/NCF<sub>FGF(18,22,24)</sub> cells expressing and secreting high levels of bFGF did not have significantly higher p21<sup>WAF1/CIP1</sup> protein levels than control cells. A 24 hour incubation with exogenous recombinant 18 kD bFGF induced an increase in the levels of p21<sup>WAF1/CIP1</sup> protein in MCF-7/N2 and in MCF-7/ $\Delta$ A<sub>FGF(18)</sub> cells, in accordance with previously reported findings (8). In contrast, exogenous bFGF caused only a minimal increase in the levels of p21<sup>WAF1/CIP1</sup> in MCF-7/NCF<sub>FGF(18,22,24)</sub> cells. These data suggest that unlike the inhibition of MCF-7 cells by exogenous 18 kD bFGF (8), the inhibition of proliferation by overexpression of nucleus-localizing bFGF species in MCF-7 cells is not mediated through induction of p21<sup>WAF1/CIP1</sup>.

We investigated the effects of bFGF on another heat stable cyclin dependent kinase inhibitor, p27<sup>Kip1</sup>. The protein levels of p27<sup>Kip1</sup> were elevated in MCF-7/NCF<sub>FGF(18,22,24)</sub> and MCF-7/ $\Delta$ A<sub>FGF(18)</sub> cells at baseline (Figure 9B). Under the conditions described, exogenous recombinant 18 kD bFGF also caused a substantial increase in the levels of p27<sup>Kip1</sup> in MCF-7/N2 cells after a 24 hour incubation unlike the preliminary observations reported under different conditions (8). These data suggested that p27<sup>Kip1</sup> may play a role in the inhibition of MCF-7/NCF<sub>FGF(18,22,24)</sub> and MCF-7/ $\Delta$ A<sub>FGF(18)</sub> cell proliferation at baseline. To determine the roles of these inhibitors on the mechanism of cell cycle inhibition in these cells, we investigated the effects of bFGF overexpression on events in the G<sub>1</sub> phase of MCF-7/NCF<sub>FGF(18,22,24)</sub> cells.

We carried out Western blots on proteins responsible for the transition of cells from G<sub>1</sub> to the S phases of the cell cycle. Figure 9C demonstrates that the levels of cyclin D<sub>1</sub>, cyclin E, cdk4, the activation state of cdk2 and the hyperphosphorylation of Rb were not different in MCF-7/NCF<sub>FGF(18,22,24)</sub> cells from those of MCF-7/N2 cells. In addition, while exogenous recombinant bFGF induced increases in the levels of cyclin D<sub>1</sub>, cyclin E and cdk4, the disappearance of the rapidly migrating, active form of cdk2 and the dephosphorylation of Rb in MCF-7/N2 cells, changes described previously in MCF-7 cells (8), it had no effect on the levels or states of these proteins in MCF-7/NCF<sub>FGF(18,22,24)</sub> cells. These data suggest that the mechanism of inhibition of MCF-7 cell proliferation by bFGF in MCF-7/NCF<sub>FGF(18,22,24)</sub> cells is not mediated by inactivation of cyclin dependent kinases and dephosphorylation of Rb but is due most likely to direct, undefined effects of nuclear localizing bFGF species. These effects are the subject of ongoing investigations.

Figure 9



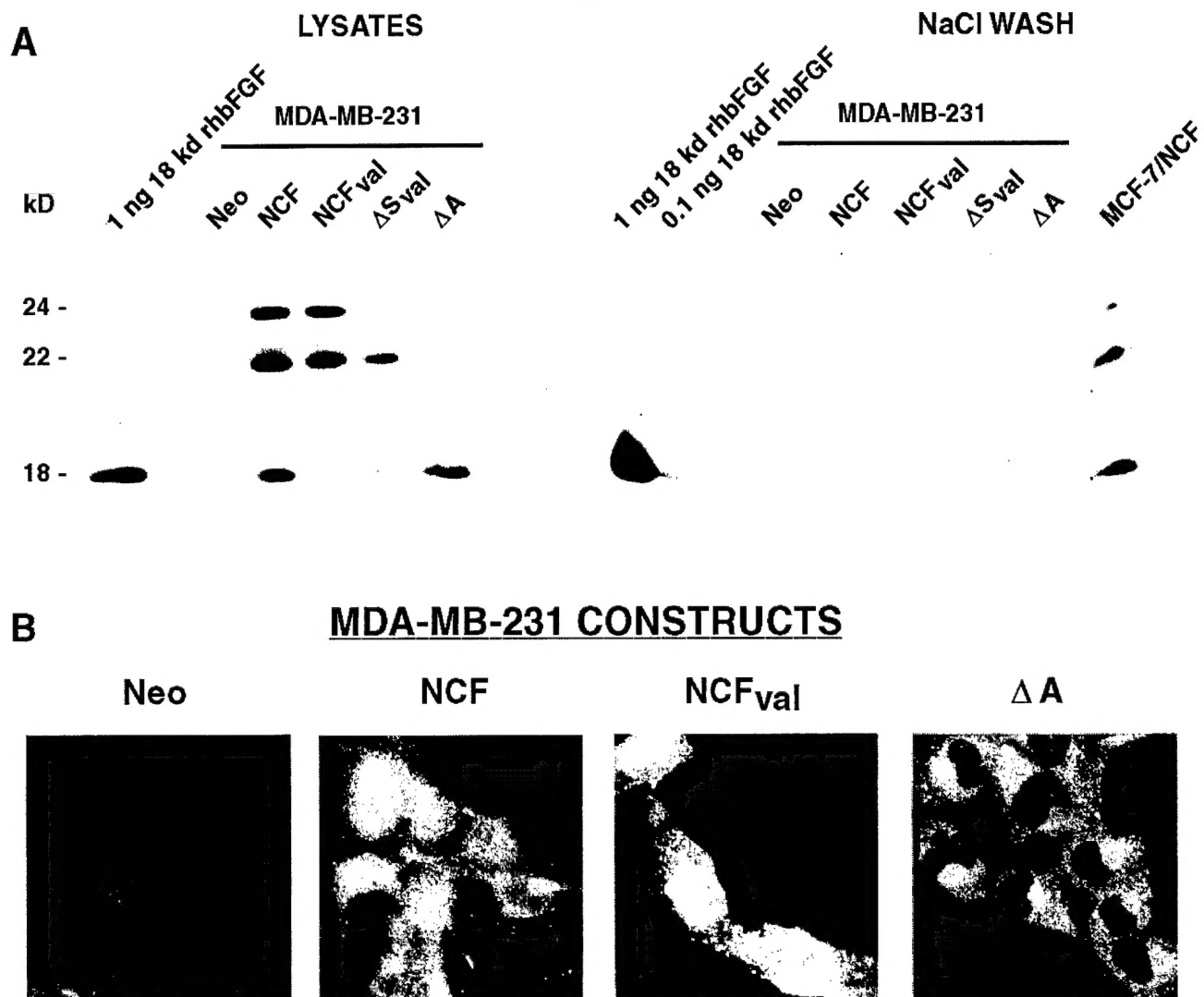
**Figure 9.** A. Phosphorylation of Histone H1 by reconstituted cyclin E/cdk2 complexes. Histone H1 was phosphorylated in an immune complex kinase assay, as described, by mink lung epithelial cell Mv1Lu lysates before (control) and after reconstitution of cyclin E/cdk2 complexes (induction). The kinase activity was assayed in the presence of lysates obtained from MCF-7 and MCF-7/N2 cells treated with medium (- bFGF) or bFGF 10 ng/ml (+ bFGF) for 24 hours or from untreated MCF-7/NCF cells, to demonstrate the transfer of a heat stable kinase inhibitory activity. B. Western immunoblots of lysates from MCF-7/N2 cells treated with 0, 0.1, 1.0, 3.0 and 10. ng/ml bFGF, MCF-7/NCF and MCF-7/ΔA cells treated with 0 and 10. ng/ml bFGF for 24 hours stained with monoclonal antibodies to p21<sup>WAF1/CIP1</sup> or p27<sup>Kip1</sup>. C. Western blots of lysates from bFGF-treated MCF-7/N2 and MCF-7/NCF cells with antisera to cyclin D<sub>1</sub>, cdk4, cyclin E, cdk2 or monoclonal antibody to Rb. Subconfluent cultures were incubated with bFGF 10 ng/ml for 24 hours, lysed, electrophoresed and analyzed by Western blot.



# Overexpression of bFGF in MCF-7 and MDA-MB-231 cells partially reverses malignant behavior

To determine if the effects of bFGF on proliferation and transformation were similar in cells at different stages of dedifferentiation, we transfected MDA-MB-231 cells with bFGF constructs expressing the 18 kD ( $\Delta A$ ), the 22 and 24 kD species (we mutated the 18 kD initiation site from methionine to valine to make NCF<sub>val</sub>) and the three species combined (NCF) and selected with G418 by virtue of the Neo gene in the vector. Figure 10 demonstrates that the cells expressed the expected species. NaCl washes of cell monolayers demonstrated that these cells have lost the capacity to export bFGF (figure 10A). Subcellular localization with immunofluorescence tagging confirmed that the various constructs expressed bFGF species that localized as expected (figure 10B).

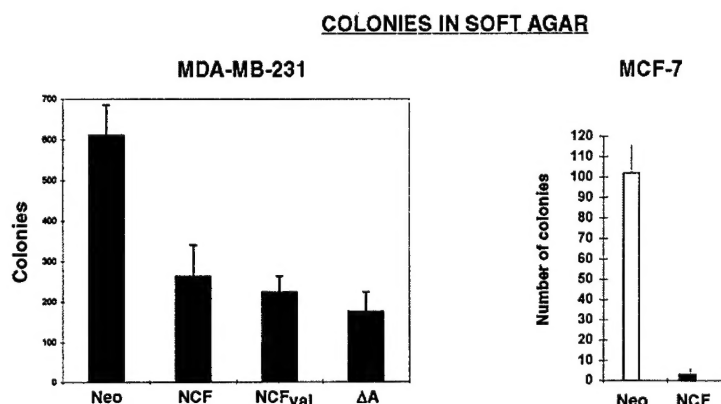
Figure 10



**Figure 10.** A. Western immunoblot of lysates and 2 M NaCl washes of MDA-MB-231 cells transfected with the various bFGF vectors and selected in G418. B. Immunofluorescence photographs taken at 400x magnification of MDA-MB-231 cells transfected with the neo vector, with NCF containing both cytoplasm and nucleus-localizing bFGF species, with NCF<sub>val</sub>, containing only the 22 and 24 kD species and  $\Delta A$ , expressing only the 18 kD species that lacks a nuclear localization sequence.

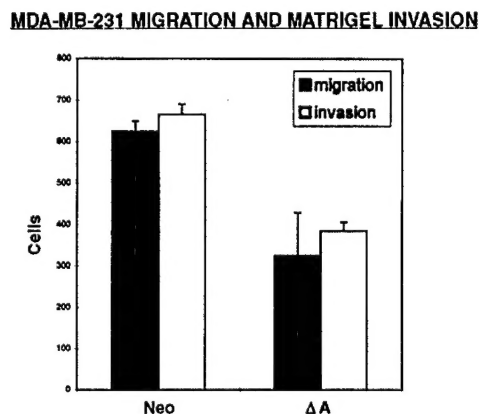
The proliferation of the cells and the clonogenic potential as determined by day 12 $\pm$ 2 colony formation on tissue culture plates was not affected by the expression of bFGF. These cells are far more dedifferentiated than MCF-7 cells and have lost the capacity to respond to both exogenous recombinant bFGF receptor-mediated signalling as well as internal bFGF-mediated cell cycle inhibition. We assessed the effects of bFGF expression on the transformed phenotype of these cells using two assays: colony formation in soft agar (13) and migration and invasion through Matrigel in a modified Boyden chamber. Figure 12 demonstrates that expression of all isoforms of bFGF in MDA-MB-231 cells substantially increased the colony forming capacity of these cells in soft agar. Similarly, we assayed MCF-7/NCF cells using the same assay. Figure 11 demonstrates that bFGF also inhibits anchorage-independent growth in these cells as well, but the effect appears to be far more pronounced. Invasion and motility were assayed in the same Boyden chamber with and without Matrigel in the various MDA-MB-231 bFGF constructs. MDA-MB-231/ $\Delta$ A cells had both less migration at 4 hours and invasion at 8 hours than vector transfected controls. The expression of any construct containing nuclear localizing bFGF species did not affect either migration or invasion.

Figure 11



**Figure 11.** Basic FGF expression inhibits anchorage-independent growth of breast cancer cells. Cells were incubated in 0.3% Bacto agar in DMEM, 7% FCS for 12 days (MDA-MB-231 cells) and 7 days (MCF-7 cells) and colonies containing  $\geq 30$  cells were counted at 100x magnification.

Figure 12



**Figure 12.** Expression of 18 kD bFGF inhibits migration and invasion capacity in MDA-MB-231 cells. Cells were incubated in a modified Boyden chamber inserts with ethylene terephthalate filters with 8  $\mu$ m pores in 24 well tissue culture dishes without (migration) or with Matrigel (invasion). Cells were incubated for 4 hours for migration and 8 hours for invasion, the filters were stained with methylene blue and the cells were counted at 100x magnification.

## CONCLUSIONS

Our data demonstrate that expression of bFGF inhibits proliferation of MCF-7 cells and restricts the cells in the G<sub>1</sub> phase of the cell cycle. MCF-7 cells, a relatively differentiated malignant mammary epithelial cell line that have already lost the capacity to synthesize bFGF are still able to secrete all isoforms of bFGF expressed from a vector. Exported bFGF inhibits binding by exogenous bFGF, probably by competitive inhibition. The high molecular weight form of bFGF appears to downregulate FGFR4 both at the membrane and in cellular lysates. The effects of overexpressing high molecular weight bFGF are to inhibit phosphorylation of FGFR1, MAP kinase signalling and to prevent modulation of the G<sub>1</sub> cyclin dependent kinase inhibitors and Rb phosphorylation. Thus, the cell cycle inhibition observed by bFGF expressed in MCF-7 cells may be mediated by other, yet undefined, possible intranuclear mechanisms.

We also demonstrate for the first time that the expression of bFGF appears to partially reverse the malignant phenotype in two breast cancer cell lines distantly separated on the spectrum of dedifferentiation. Both MCF-7 and MDA-MB-231 cells expressing bFGF have a decreased capacity for anchorage-independent growth and MDA-MB-231 cells expressing 18 kD bFGF have a decreased capacity to migrate and invade through Matrigel. These data demonstrate, for the first time, that the reported association between the presence of bFGF and less malignant behavior in breast cancer cells may be caused by bFGF.

**REFERENCES**

1. Burgess, W. H., and Maciag, T. The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem.*, 58:575-606, 1989.
2. Folkman, J. The role of angiogenesis in tumor growth. *Seminars in Cancer Biol.* 3:65-71, 1992.
3. Luqmani, Y. A., Graham, M., and Coombes, R. C. Expression of basic fibroblast growth factor, FGFR1 and FGFR2 in normal and malignant human breast, and comparison with other normal tissues. *British J. Cancer*, 66:273-280, 1992.
4. Anandappa, S. Y., Winstanley, J. H.R., Leinster, S., Green, B., Rudland, P. S., and Barraclough, R. Comparative expression of fibroblast growth factor mRNAs in benign and malignant breast disease. *British J. of Cancer*. 69:772-776, 1994.
5. Gospodarowicz, D., Massoglia, S., Cheng, J., Lui, G. M., and Bohlen, P. Isolation of bovine pituitary fibroblast growth factor purified by fast protein liquid chromatography (FPLC). Partial chemical and biological characterization. *J. Cell. Physiol.*, 122:323-333, 1985.
6. McLeskey, S. W., Ding, I. Y. F., Lipman, M. E., and Kern, F. G. MDA-MB-134 breast carcinoma cells overexpress fibroblast growth factor (FGF) receptors and are growth-inhibited by FGF ligands. *Cancer. Res.*, 54:523-530, 1994.
7. Fenig, E., Wieder, R., Paglin, S., Wang, H., Persaud, R., Haimovitz-Friedman, A., Fuks, Z., and Yahalom, J. Basic fibroblast growth factor confers growth inhibition and Mitogen-activated Protein Kinase activation in human breast cancer cells. *Clinical Cancer Res.* 3:135-142, 1997.
8. Wang, H., Rubin, M., Fenig, E., DeBlasio, T., Mendelsohn, J., Yahalom, J., and Wieder, R. Basic FGF causes growth arrest in MCF-7 human breast cancer cells while inducing both mitogenic and inhibitory G<sub>1</sub> events. *Cancer Research* 57:1750-1757, 1997.
9. Lai, L. C., Sirah, A. K., Erbas, H., and Lennard, T. W. J. Relationship between basic fibroblast growth factor and transforming growth factor-beta 2 in breast cyst fluid. *J. Clin. Endoc. & Metab.* 80:711-714, 1995.
10. Gomm, J. J., Smith, J., Ryall, G. K., Baille, R., Turnbull, L., Coombes, R. C. Localization of basic fibroblast growth factor and transforming growth factor  $\beta$ 1 in the human mammary gland. *Cancer Res.* 51:4685-4692, 1991.
11. Wieder R, Fenig E, Wang H, Wang Q, Paglin S, Menzel T, Gabrilove J, Fuks Z, Yahalom J. (1998) Overexpression of basic fibroblast growth factor in MCF-7 human breast cancer cells: lack of correlation between inhibition of cell growth and MAP kinase activation. *J. Cellular Physiology* 177:(in press).
12. Renko, M., Quarto, N., Morimoto, T., and Rifkin, D. B. Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell. Physiol.*, 144:108-114, 1990.
13. Wieder, R., Wang, H., Shirke, S., Wang, Q., Menzel, T., Feirt, N., Jakubowski, A. A., and Gabrilove, J. L. Low level expression of basic FGF upregulates Bcl-2 and delays apoptosis, but high intracellular levels are required to induce transformation in NIH 3T3 cells. *Growth Factors*, 15:41-60, 1997.
14. Jaye, M., Schlessinger, J., and Dionne, C. A. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochimica et Biophysica Acta*, 1135:185-199, 1992.
15. Bellot, F., Crumley, G., Kaplow, J. M., Schlessinger, J., Jaye, M., and Dionne, C. A. Ligand-induced transphosphorylation between different FGF receptors. *The EMBO Journal*, 10:2849-2854, 1991.